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Degradation of diesel with microorganisms in rhizosphere of *Carex phacota* Spr.

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***Abstract*—Because of widespread use of petroleum, the Huangpu-Yangtze River estuary (HYRE) wetland has been polluted by accidental spills. The technology to degrade these compounds is a major goal of environmental research. This study isolated oil-degrading microorganisms from oil contaminated wetland in the HYRE. Three bacterial strains were discovered, and identified by sequencing their 16S rDNA genes. Two of them were *Pseudomonas* and the other one was *Alcaligenes*. Diesel biodegradation potential of these indigenous oil-degrading microorganisms(HPM) and HPM co-metabolize with the native plant *Carex phacota* Spr.(CPS) were assessed. During the 60-day experiment, soil samples were collected and analyzed periodically to determine the residual diesel content and microbial populations. The results showed that the oil-degrading microorganisms isolated from the HYRE wetland had a certain degradation effect on diesel. Within 60 days, the relative degradation rates of microorganisms were 8.05~36.59%; and under the combined effects of microorganisms and plants, the degradation rates of diesel could reach 28.28~52.94% at different concentration of diesel. They all play a good role on the *n*-alkanes within the range of C_{18}~C_{24} components of diesel. Results indicated that the oil-degrading bacteria isolated from the HYRE wetland have a certain degradation effect on diesel. The co-metabolize of plants and microbes would increase the diesel biodegradation rate. The plants and the oil-degraders in wetland could be reasonably matched to control the diesel pollution of wetland sediment.**

Keywords—wetlands; diesel; degradation; microbe; Carex phacota Spr.

I. INTRODUCTION

Huangpu River-Yangtze River as a main water transport channel of Shanghai had been polluted by oily pollutants[1]. Oils were the main pollutants in the Huangpu-Yangtze River estuary (HYRE) sediment[2]. The oily water discharged by ships leads to the pollution of HYRE water and the coastal wetlands. Bioremediation and phytoremediation, the use of microorganism and plants to remove or degrade contaminants, has been extensively researched in recent years[3,4].

Phytoremediation of organic pollutants depends on plant-microbe interactions in the rhizosphere, grass species have frequently been suggested as effective plants for treating hydrocarbon-contaminated soils due to their fibrous root systems, which have a large surface area per unit volume near the soil surface[5], and the extent and intensity of such rhizosphere effects are likely to decrease with increasing distance from the root surface[6]. This enhanced dissipation of pollutants in planted soils might be derived from increased microbial activity and plant-released enzymes. During the experimental period, a relatively large amount of phenolic compounds, high microbial activity, and high peroxidase activity were detected in planted soils[7]. The rhizoremediation of many grass species, such as alfalfa, rape, vetch and mustard, mulberry, on the fate of hydrocarbons has been studied[8]. Mustard and vetch fostered the removal of PHCs from soil. The two crops elicited the greatest degradative root activities and sustained particularly great populations of rhizosphere bacteria that are known as hydrocarbon degraders[8,9]. Rasolomanena and Balandreau found that a bacterium, *Bacillus-sp*, isolated from oil-contaminated paddy soil could grow in the oil residues in the presence of rice root exudates. This indicated that the rice root system promoted the specific micro-organisms to eliminate oil residue[9]. However, relatively few wetland plant species have been studied for phytoremediation purposes.

This study (i)screen the oil-degraders from the oil-contaminated soil of the HRYE wetlands and make identification, (ii)evaluated the diesel degradation ability of indigenous oil-degrading microorganisms, (iii) assessed the diesel degradation potential if the oil-degraders co-metabolize with *Herba Caricis Phacotae* (*Carex phacota* Spr., CPS), which is a native plant of HYRE wetland.（iv）the change in the number of microorganisms in the soils, (v) component changes after biodegradation.

II. MATERIALS AND METHODS

A. Soils and diesel

1) The soil used to screen oil-degrading microorganisms was collected from the surface (1~10cm depth) sediment of HYRE wetland (N31°23’5.4’’, E121°30’28.3’’).

2) The soils with no previous history of contaminants were collected from field in the east campus of Shanghai University.

3) Diesel fuel, $0^{\#}$, was bought from Minghe petrol station of Sinopec in Baoshan district.

B. Experiment microbes

1) Isolation and screening cultures

a. Mineral salts medium (MSM)(g/L): K_2HPO_4 (2g), KH_2PO_4 (0.5g), NaCl (0.5g), NH_4Cl (0.5g), $MgSO_4$ (0.2g), $CaCl_2$ (10mg), pH 7.2, containing different concentration of diesel.

b. Enrichment medium(EM): beef extract, peptone culture medium and glucose medium.

c. Preservation medium(PM): beef extract, peptone medium.

2) Isolation, maintenance and identification of bacterial strains

Oil-degrading microorganisms were isolated by direct plating dilutions of oil comtaminated soils on MSM containing diesel as the sole carbon and energy sources[10]. The domestication process repeated several times with the increase of the diesel concentration (until 1ml per 100ml MSM). Isolates were plated on the MSM agar containing diesel. Discrete colonies were picked and purified two times on EM. The strains were kept on PM plate at 4℃. The oil-degraders were identified by cloning and sequencing 16S rDNA genes[11] in Sangon Biotech(Shanghai) Co.,Ltd.

3) Bacterial suspension preparation

The efficient oil-degraders were inoculated into EM, 30 ℃ and cultivated to logarithmic phase. The bacteria were collected and suspended in saline. The absorbance (OD_{660}) of bacterial suspension was adjusted to 1.5.

C. Experiment plants

The plants used to treat diesel-contaminated soils was *Carex phacota* Spr.(CPS), which was transplanted from the shore of the Huangpu river.

D. Pot experiments design

The soil sample were packed into experimental pots, and mixed with diesel at the concentration of 5000, 10000, 15000 and 20000 $mg \cdot kg^{-1}$, then laid in the open air for one week to evaporate the unstable components in the diesel sample.

The plants with similar biomass were transplanted into the pots, along with an unplanted control. The experiment was divided into three groups. 25 ml bacterial suspension was mixed into the planted soils as the experiment samples(CPS-Microbes). The second group was only added 25ml bacterial suspension as the microbes samples. The control group was unplanted and un-inoculated soils. All the pots were placed in the open air for 60 days.

E. Components analysis

Diesel contaminants of every pot were evaluated at the experiment beginning, and residual diesels were determined after 60 days. Diesel contaminants had been extracted by ultrasonic method (5g dry soil, 30ml of dichloromethane: hexane 1:1) and evaluated by gravimetric method [12].

The components of the residual diesels were analyzed with Gas Chromatography (GC). The GC analysis was on an Agilent 7890 and FID detector [8].

F. Microbial plate counts

The number of microorganisms is monitored by standard plate counts. The microbial population was extracted from the soils defined as "rhizosphere soils" which firmly attached to the plant roots[13].

III. RESULTS AND DISCUSSION

A. Isolation and identification of bacterial strains

Three strains of aerobic bacteria were isolated from the oil-contaminated soil. The macroscopic (color, form and colony size) characteristics of strains in the experimental are as follows:

M1: The colony is yellow, translucent, with irregular circular convex. Its surface is smooth and shiny, and its diameter is 0.5~2mm. Metabolites make medium blue-green.

M2: The colony is milky white, opaque, with circular convex. Its surface is smooth and shiny, and its diameter is 2~3mm.

M3: The colony is white, opaque, with circular convex. Its surface is smooth and shiny, and its diameter is 0.5~1mm.

They were further identified by partial sequencing their 16S rDNA genes. Among the three isolates obtained, two were identified as *Pseudomonas* (M1 and M3) and one as *Alcaligenes*(M2). There have been some studies about their application to bioremediation In the further study, the composition of the three bacterial strains were called HPM.

B. Diesel degradation by indigenous oil-degraders and CPS -Microbes

Reduction of diesel concentrations was measured in separate microcosms after 60 days(Figure 1). Three groups of microcosms were subjected to the same amendment regimes. The microbes samples showed a good role on diesel degradation. The growth of *Carex phacota* Spr. in diesel-contaminated soils indicated a certain tolerance to diesel, and the degradation effect of plant-microbes generally exceeded the effect of microorganisms.

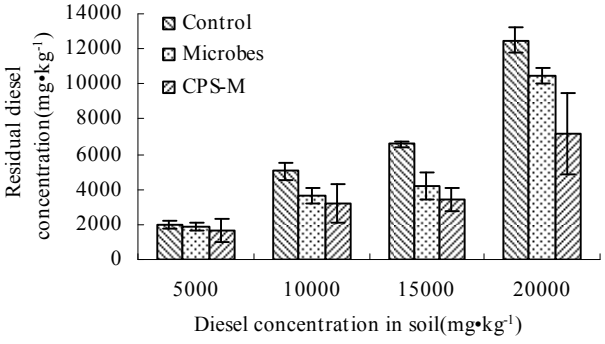

Figure 1. Residual diesel concentration after 60 days

The diesel degradation rates of microorganisms and plant-microbes at various levels of diesel are shown in Figure 2. The biodegradation rates of planted samples are higher than that of unplanted ones, findings supported contaminants removal data in some studies[14]. The CPS-M caused a further reduction on diesel contaminant, rather than

only inoculated microbes microcosm and control microcosm. It meant the oil-degrader played a certain role on the degradation of diesel, the degradation rates were 8.05~36.59% after 60 days. The microcosm vegetated CPS, it could served as nutrient to the microcosm, the degradation effects increased in response to the nutrient addition. The diesel degradation rates reached 28.28~52.94% under the effect of CPS-M.

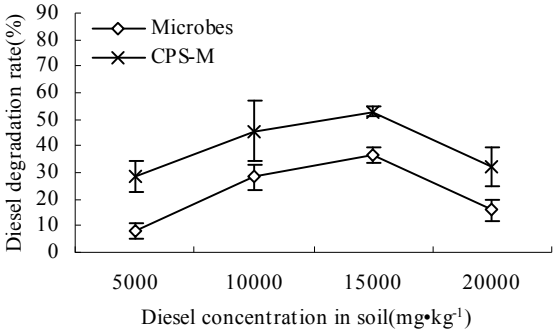

Figure 2. Diesel degradation rates after 60 days

Simultaneously, the biodegradation rates are influenced by the diesel concentration in soils. Under test conditions, the microcosms tend to utilize diesel as nutrient, and the optimal diesel concentration is 15000 mg·kg^{-1}(Figure 2). As the increasing of diesel concentration, the degradation rates increased, showed a peak value at 15000 mg·kg^{-1}, then it decreased at the increasing concentration. The higher concentration of contaminant could be disincentive to the growth of CPS. A hydrophobic coat of oil, which covers the root, may disrupt the root nutrient uptake. Base on the 15000mg·kg^{-1} diesel soils, the concentration of the total petroleum hydrocarbons (TPH) had been reduced from 12785 to 3441.59 mg·kg^{-1} (75.81%) in the planted soils after 2-month experiment, while it reduced to 4166.96 mg·kg^{-1}(67.41%) in the unplanted sample.

C. Alteration of microbial populations

The change in the microbial populations of each pot with time was found. An overall reduction with time in the microbial populations of each pot was found at various diesel concentrations because of the presence of diesel in the soils. But it flourished in the vegetated soils a month later in Figure 3c. Contrastly, it reduced all the time in the control soils and the microbe soils(Figure 3a, 3b).

On the basis of others results, the microorganisms which decompose hydrocarbons might be the dominant microbial population at the end of the process[4]. Generally the number of microorganisms decreased few in the vegetated CPS soils. It confirms that high endophytic populations have been associated with plant. Oil-degrading microbes can co-metabolize oil using plant root compounds as primary substrates. The total culturable microflora were higher in the rhizospheric than non-rhizospheric soil. In a study, under the influence of plant, the density of the bacterial community increased the first year and stayed constant in the PAHs polluted soil. Hydrocarbon degradation is believed to occur through a rhizosphere effect plants exude organic compounds through their roots, which increases the density, diversity and activity of specific microorganisms in the surrounding rhizosphere, which in turn degrade hydrocarbons[9].

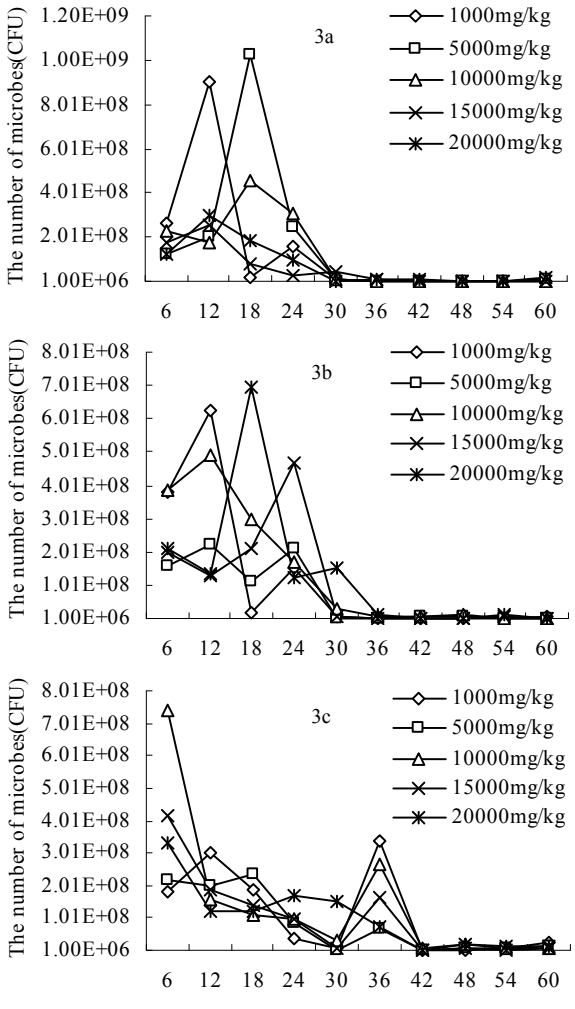

Figure 3. The change in the number of microorganisms in the control soils(3a), the microbes soils(3b) and the CPS-M soils(3c)

Although the counts of microorganisms alone do not represent an accurate measurement of the actual biodegradation combine with plant CPS, it is suggested that CPS contribute to the growth of oil-degraders population in contaminated soils. The microorganisms can be used as a good indicator for oil phytoremediation.

D. Degradation characteristics

The degradation characteristics of oil-degraders and it combine with CPS were analyzed. Distribution characteristics of *n*-alkanes in the soils at 10000 mg·kg^{-1} diesel concentration after 60 days were shown in Figure 4.

It indicates that the oil-degraders isolated from HYRE estuary wetland have made changes in the composition on the *n*-alkanes component of diesel(Figure 4). The plant

Carex phacota Spr. collected from HYRE wetland combined with the oil-degraders made a further degradation on the *n*-alkanes. Based on the levels of 10000mg·kg^{-1} soil, after 60 days, the diesel biodegradation rate on the role of oil-degraders was 28.28% in the un-planted pots, that on the role of CPS-M was 45.58%. The microbes have played a remarkable role on the *n*-alkanes C_{19} especially. Simultaneously CPS-M have a degradation effect on the range of C_{19}~C_{21} *n*-alkanes.

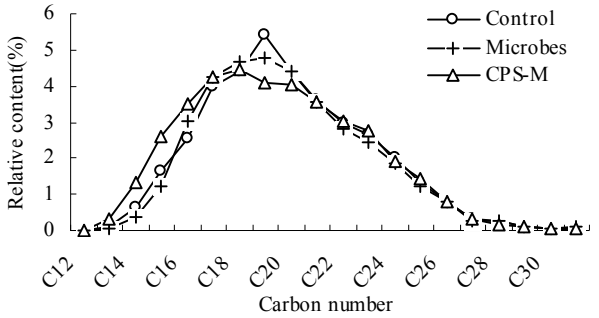

Figure 4. Distribution characteristics of *n*-alkanes at 10000 mg·kg^{-1} diesel concentration in the soils after 60 days

IV. CONCLUSIONS

Results of this study suggested that the intrinsic bacteria along Huangpu-Yangtze shorelines have the ability to degrade diesel. We vegetated indigenous plant *Carex phacota* Spr. in the soil to metabolize with intrinsic oil-degraders, got good degradation results. Because the set-up of this experiment emulated a natural environment, it is thought that the application of planting the *Carex phacota* Spr. species to served as nutrients and contributed to the microbial activity and peroxidase activity in the soil, but it should be tested before any decision is made. It is believed that this test provides a database for further evaluations and provides a support to design optimal treatments in wetland ecological restoration field.

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